Veterinary Immunology and Immunopathology 137 (2010) 327–331

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Technical report

Molecular cloning, characterization and *in vitro* expression of SERPIN B1 of bighorn sheep (*Ovis canadensis*) and domestic sheep (*Ovis aries*), and comparison with that of other species

Renuka Subramaniam[a], Rohana P. Dassanayake[a], Junzo Norimine[a], Wendy C. Brown[a,b], Donald P. Knowles[a,c], Subramaniam Srikumaran[a,*]

[a] *Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, USA*
[b] *School for Global Animal Health, Washington State University, Pullman, WA 99164-7040, USA*
[c] *Animal Disease Research Unit, USDA Agricultural Research Service, Pullman, WA 99164-6630, USA*

ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form 23 March 2010
Accepted 28 May 2010

Keywords:
Bighorn sheep
Domestic sheep
SERPIN B1
Mannheimia haemolytica

ABSTRACT

Mannheimia haemolytica infection results in enhanced PMN-mediated tissue damage in the lungs of bighorn sheep (BHS) compared to that of domestic sheep (DS). SERPIN B1 is an inhibitor of PMN-derived serine proteases. It prevents lung tissue injury by inhibiting the serine proteases released as a result of PMN lysis and degranulation. It is conceivable that PMNs of BHS exhibit decreased quantity and/or activity of SERPIN B1 which results in enhanced tissue injury and decreased bacterial clearance in pneumonic lungs of BHS. The objective of this study was to clone and express SERPIN B1 of BHS and DS, and develop antibodies to facilitate quantification of SERPIN B1. The 1,134 bp cDNA of SERPIN B1 of BHS and DS encodes a polypeptide of 377 amino acids. SERPIN B1 of BHS and DS exhibits 100% identity at the nucleotide and amino acid levels. The amino acid sequence of ovine (BHS/DS) SERPIN B1 displays 69%, 71%, 74%, 78% and 80% identity with that of rats, dogs, mice, humans and horses, respectively. Ovine SERPIN B1 expressed in *Escherichia coli* was used to develop polyclonal antibodies in mice. Western blot analysis revealed the specificity of these antibodies for ovine rSERPIN B1.

The bighorn sheep (BHS, *Ovis canadensis*) population in North America has dramatically declined over the years, from an estimated 2 million in the 1800s to less than 70,000 at present (Buechner, 1960). Pneumonia is the disease that contributed the most to the decline of BHS (Coggins, 1988). *Mannheimia haemolytica* is an important etiological agent of pneumonia in BHS, domestic sheep (DS, *Ovis aries*) and other ruminants. Leukotoxin produced by *M. haemolytica* is cytolytic to all subsets of leukocytes, but PMNs are the most susceptible subset (Jeyaseelan et al., 2002). Leuotoxin-induced cytolysis and degranulation of PMNs are responsible for the acute inflammation and lung damage characteristic of this disease (Slocombe et al., 1985). Experimental inoculation of *M. haemolytica* results in rapid death of BHS, but not DS. Severe lung tissue injury and decreased bacterial clearance are observed in BHS, but not in DS (unpublished data), indicating that BHS are much more susceptible to *M. haemolytica*-caused pneumonia than DS (Foreyt, 1989, 1994; Dassanayake et al., 2009). The cellular and molecular mechanisms underlying the differential susceptibility of BHS and DS are not clear.

Serine Protease Inhibitor B1 (SERPIN B1), a member of the clade B serpins, is the most effective inhibitor of PMN-derived proteases such as neutrophil elastase, cathepsin G and proteinase-3. SERPIN B1, also known as monocyte neutrophil elastase inhibitor (MNEI), is abundant in sec-

* Corresponding author at: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040, USA. Tel.: +1 509 432 4572; fax: +1 509 335 8529.
E-mail address: ssrikumaran@vetmed.wsu.edu (S. Srikumaran).

0165-2427/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2010.05.017

```
atggagcagctgagcgcagcgaacacccgcttcgccgtggacctgttccgcaccctggcc
M  E  Q  L  S  A  A  N  T  R  F  A  V  D  L  F  R  T  L  A  20
gagcacaatcctgccggaaacatcttcctctccccttcagcatctcgtcagcgctggcc
E  H  N  P  A  G  N  I  F  L  S  P  F  S  I  S  S  A  L  A  40
atggtctttctgggggccagaggcgacaccgcggcacagctttccaaggttctccatttc
M  V  F  L  G  A  R  G  D  T  A  A  Q  L  S  K  V  L  H  F  60
gaaggagttgagattcattcaggatttcagagtctgaatgctgatatcaacaagcatggt
E  G  V  E  I  H  S  G  F  Q  S  L  N  A  D  I  N  K  H  G  80
gctccctacactctgaaactcgccaacaggttattcggagagaaaagctacaatttcctc
A  P  Y  T  L  K  L  A  N  R  L  F  G  E  K  S  Y  N  F  L  100
cctgagttcttagcttcaatgcagaaaacgtacagtgctgagctggccagcgtggacttt
P  E  F  L  A  S  M  Q  K  T  Y  S  A  E  L  A  S  V  D  F  120
ctgcgggcctccgaggaggcgaggaggagcataaacgagtgggtcaaagggcagacagga
L  R  A  S  E  E  A  R  R  S  I  N  E  W  V  K  G  Q  T  G  140
gggaaaattccggagctgttggcctcaggtatggttgacagcttgacgaggctggtgctg
G  K  I  P  E  L  L  A  S  G  M  V  D  S  L  T  R  L  V  L  160
gtgaacgccatctatttcaaagggagctggcaggagaagttcatggtggaggccaccaag
V  N  A  I  Y  F  K  G  S  W  Q  E  K  F  M  V  E  A  T  K  180
gatgcgcctttcagactgaataagaaagaaacaaaaacggtgaagatgatgtatcagaag
D  A  P  F  R  L  N  K  K  E  T  K  T  V  K  M  M  Y  Q  K  200
aagaagtttcccttcggctacatcaaggaccttaagtgccgggtgctggagctgccctac
K  K  F  P  F  G  Y  I  K  D  L  K  C  R  V  L  E  L  P  Y  220
gagggcaaggacctcagcatggtcatcctgctgccggacgacatccaggacgaggccacg
E  G  K  D  L  S  M  V  I  L  L  P  D  D  I  Q  D  E  A  T  240
gggctgaagaagattgagcaacagctgactctggagaagctgcgggagtggacccaaccc
G  L  K  K  I  E  Q  Q  L  T  L  E  K  L  R  E  W  T  Q  P  260
gagaacctggacctccttgaggtcagggtccagctgcccaggttcaagctggaggagagt
E  N  L  D  L  L  E  V  R  V  Q  L  P  R  F  K  L  E  E  S  280
tatgacctcagcgagcccctggcccgcctgggtgcacgggatctcttcagcagcaaggcg
Y  D  L  S  E  P  L  A  R  L  G  A  R  D  L  F  S  S  K  A  300
gacctgtccggcatgtcaggggccagcgacctcttcatatccaaggtggtccacaagtct
D  L  S  G  M  S  G  A  S  D  L  F  I  S  K  V  V  H  K  S  320
gtcgtggacgtgaacgaggagggcacggaggcggcggctgccacgggggccatcgcccag
V  V  D  V  N  E  E  G  T  E  A  A  A  A  T  G  A  I  A  Q  340
ttcgctatgctgatgccggaggaggagttcgttgccgaccacccccttcatcttcttcatc
F  A  M  L  M  P  E  E  E  F  V  A  D  H  P  F  I  F  F  I  360
cggcacaagccctcctcgcacatcctgtttctaggcaggctttcctccccatag
R  H  K  P  S  S  H  I  L  F  L  G  R  L  S  S  P  -        377
```

Fig. 1. The nucleotide and deduced amino acid sequence of ovine SERPIN B1 cDNA. Since the nucleotide and amino acid sequences of SERPIN B1 of BHS and DS are 100% identical, we present nucleotide and amino acid sequences as ovine SERPIN B1.

ondary granules of PMNs. The lysis and degranulation of PMNs results in the release of neutrophil serine proteases into the lungs which could lead to severe tissue injury and hemorrhage. This notion is supported by the observation that instillation of rSERPIN B1 decreased hemorrhage and tissue injury following instillation of neutrophil elastase in a rat model (Rees et al., 1999). Neutrophil elastase has also been shown to cleave antibodies which results in impaired Ab-mediated phagocytosis (Fick et al., 1984), which in turn results in defective bacterial clearance. Therefore it is conceivable that decreased production and/or activity of SERPIN B1 would result in impaired inhibition of neutrophil elastase resulting in defective phagocytosis and bacterial clearance. This view is supported by the finding that when SERPIN B1-deficient mice and wild type mice were infected with *Pseudomonas aeruginosa*, SERPIN B1-deficient mice showed lower bacterial clearance and higher mortality compared to the wild type mice. Co-administration of rSERPIN B1 normalized the bacterial clearance (Benarafa et al., 2007). Therefore, it is plausible that PMNs of BHS exhibit decreased quantity and/or activity of SERPIN B1 which results in decreased bacterial clearance and enhanced tissue injury in pneumonic lungs. Our long term goal is to compare the quantity and activity of SERPIN B1 in the PMNs of BHS and DS. Towards this goal, in this study we have cloned, sequenced and expressed SERPIN B1 of BHS and DS.

Peripheral blood was collected from healthy BHS and DS. PMNs were isolated by Ficoll paque density gradient centrifugation as previously described by us (Liu et al., 2006). Total cellular RNA was extracted using TRIzol® reagent and cDNA was synthesized using Superscript III™ according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The forward primer was designed to amplify SERPIN B1 based on the available bovine SERPIN B1 sequence (BT025443) and the reverse primer was designed based on multiple alignment of SERPIN B1 of humans (NM030666), mice (AF521697), rats (XM001066387), horses (NM001081947) and unidentified sequence of DS (EE828847) in the GenBank. Sequences of the forward and reverse primers are 5′-ATG GAG CAG CTG AGC GCA G-3′ and 5′-TCA GTA TAA AAA CGA AGA AGA GAT ACC CAT GCC AGG-3′, respectively. SERPIN B1 gene was amplified using Gotaq master mix (Promega, Madison,

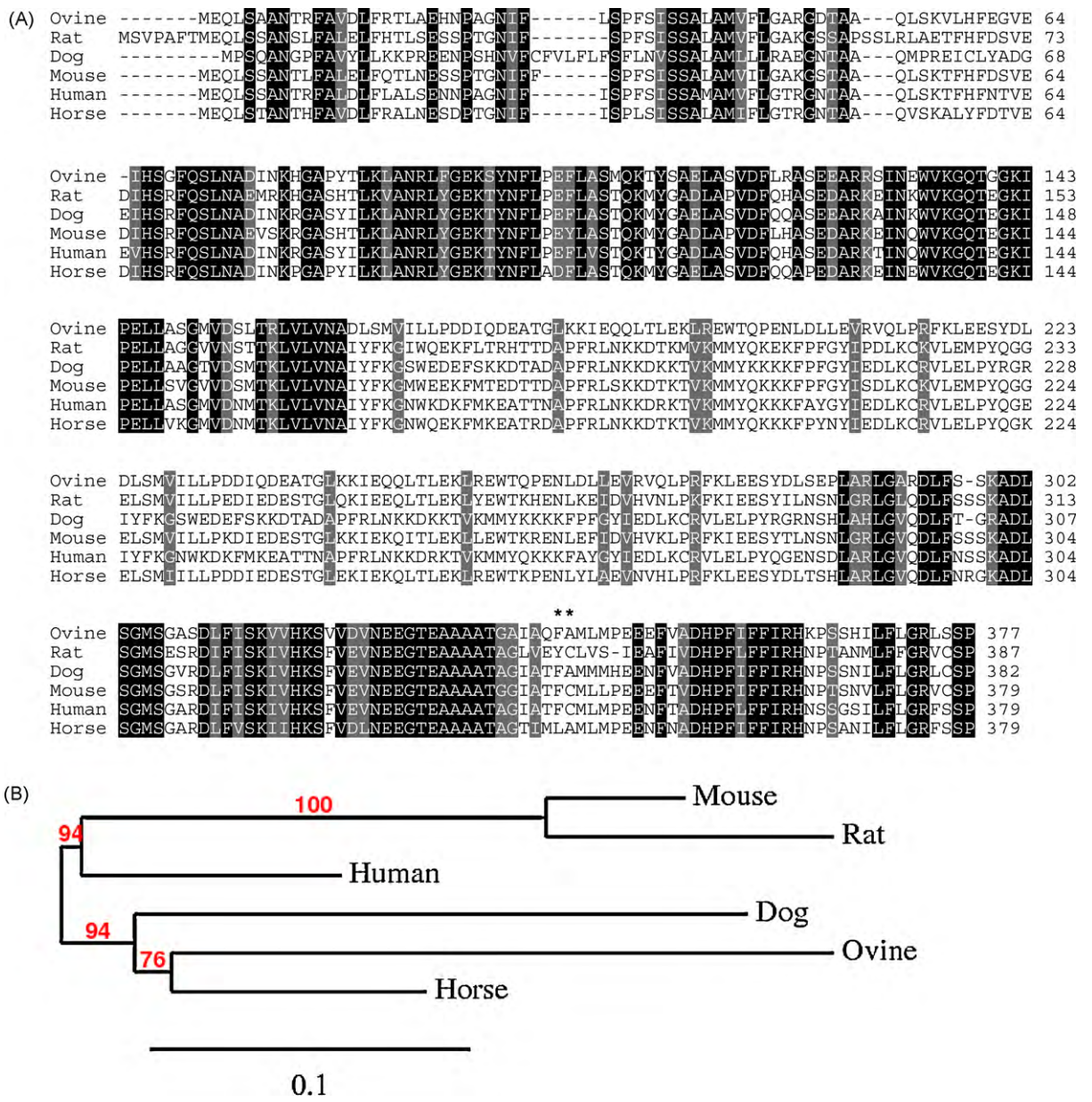

Fig. 2. (A) Comparison of the deduced amino acid sequence of ovine SERPIN B1 with those of rats, dogs, mice, humans and horses. Identical residues are presented by white text on black. (*) Designates known reactive sites in human SERPIN B1. (B) Phylogram indicating the relationship of coding sequence of ovine SERPIN B1 with those of rats, dogs, mice, horses and humans. The phylogenetic tree was generated using http://www.phylogeny.fr.

WI) according to the manufacturer's protocol. The amplicon was gel-purified and cloned into PCR®4 TOPO® vector and transformed into *Escherichia coli* (Invitrogen). Vector-specific and gene-specific colony PCR was performed to identify positive clones containing SERPIN B1 insert. Plasmids of five positive clones of BHS and DS were extracted using QIAprep Spin Mini prep kit (Qiagen, Valencia, CA) and were sequenced using Big dye™ chemistries and ABI Prism 377 DNA sequencer (Applied Bio systems, CA). The BHS (GU585525) and DS (GU585526) SERPIN B1 sequences have been deposited at GenBank. Sequences were analyzed using Vector NTI Advance TM 9.1 (Invitrogen). Clustal W2 (http://www.ebi.ac.uk) was used to align the nucleotide and amino acid sequences of BHS and DS SERPIN B1 with those of other species. A phylogram was generated using http://www.phylogeny.fr. The protein parameters were analyzed by Expasy (http://www.us.expasy.org). Since BHS and DS share 100% identity in their amino acid sequence, we decided to express rSERPIN B1 from one of these two species only (BHS). Based on the complete sequence of BHS SERPIN B1, two primers were designed to express rSERPIN B1. The forward primer and reverse primer contained sequences of *Bam*HI and *Xho*I, respectively. Forward and reverse primer sequences are 5′-CGC GGA TCC ATG

GAG CAG CTG AGC GCA GCG-3′ and 5′-CCG CTC GAG CTA TGG GGA GGA AAG CCT GCC-3′, respectively. Following double digestion with *Bam*HI and *Xho*I, the amplicon was cloned into pET-30(a) + vector (Novagen, Madison, WI) for expression of His-tagged rSERPIN B1. After screening with gene-specific PCR, the plasmid harboring correct SERPIN B1 insert was transformed into chemically competent *E. coli* BL21 (DE3) cells (Invitrogen). SERPIN B1 fusion protein was induced by adding 100 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) and was purified using nickel-NTA columns according to the manufacturer's protocol (Novagen). Anti-BHS SERPIN B1 polyclonal antibodies were generated in mice by standard immunization and bleeding protocols (Harlow and Lane, 1988). The specificity of the polyclonal antibodies was confirmed by performing Western blot analysis with rSERPIN B1.

The complete nucleotide and deduced amino acid sequence of SERPIN B1 of BHS and DS are shown in Fig. 1. Since SERPIN B1 genes of BHS and DS were 100% identical at the nucleotide and amino acid sequence levels, we present their sequence as ovine SERPIN B1 nucleotide and amino acid sequence. The 1,134 bps of cDNA of ovine SERPIN B1 encode a polypeptide of 377 amino acids. Alignment of ovine SERPIN B1 nucleotide sequence with that of dogs, rats, mice, humans and horses revealed % identity of 71, 73, 76, 78 and 80, respectively. The deduced amino acid sequence of ovine SERPIN B1 shares 69%, 71%, 74%, 78% and 80% identity with that of rats, dogs, mice, humans and horses, respectively (Fig. 2A). Fig. 2B shows the phylogram of SERPINB1 of different species. The ovine SERPIN B1 sequence exhibits a closer relationship with that of horses than those of other species. Since the bovine complete SERPIN B1 sequence is not available, it is not included in any of the analysis. Ovine SERPIN B1 has a predicted molecular weight of 42.2 kDa. In agreement with the fact that SERPIN B1 of other species is a non-glycosylated protein, our sequence analysis did not identify potential N or O glycosylation sites. The signal peptide cleavage site is not predicted by signal-P. Ovine SERPIN B1 is a non-secretory protein similar to those of humans and other species. SERPIN B1/MNEI of humans has two functional reactive sites for neutrophil elastase and chymotrypsin-like proteases (Cooley et al., 2001). Alignment of the deduced amino acid sequence revealed that ovine SERPIN B1 has one reactive site similar to that of human SERPIN B1 (Phy^{341}), and another putative reactive site (Ala^{342}) different from that of human SERPIN B1 (Cys^{344}). Ovine SERPIN B1 and that of dogs and horses have Ala instead of Cys as in humans, mice and rats. It appears that ovine SERPIN B1, like that of horses, may use adjacent reactive sites to inhibit neurtophil elastase (Remold-O'Donnell et al., 1992). Further studies are necessary to elucidate the reactive sites of neutrophil elastase.

Western blot analysis revealed the specificity of the mouse polyclonal antibodies for SERPIN B1 (Fig. 3). The pre-immune serum did not react with purified SERPIN B1 (lane 6) or any other protein from the *E. coli* lysate (lanes 4 and 5). In contrast, the immune serum revealed bands with apparent molecular weight of 46 kDa from the lysate of *E. coli* transformed with the plasmid carrying the gene for SERPIN B1 (lane 2), but not from the lysate of *E. coli* transformed with the empty vector (lane 1). The immune serum also revealed a band with apparent molecular weight of 46 kDa from the purified preparation of SERPIN B1 (lane 3). These results indicate that the immune serum recognizes SERPIN B1. The apparent molecular weight of rSERPINB1 (lanes 2 and 3) is higher (46 kDa) than the predicted molecular weight of 42.2 kDa because of the presence of His-tag with vector derived amino acids. The additional band with an apparent molecular weight of 100 kDa in lane 2 is likely to be a dimeric SERPIN B1 since a band with similar molecular weight is not seen in lane 1 (lysate of *E. coli* transformed with the empty vector). Likewise, the less intense band just below the SERPIN B1 band in lane 2 likely represents degraded SERPIN B1. The other bands with apparent molecular weight lower than 37 kDa in lanes 1–3 likely represent *E. coli* proteins bound by the immune serum because these proteins are present in the lysate of *E. coli* transformed with the plasmid carrying the gene for SERPIN B1 (lane 2) as well as the lysate of *E. coli* transformed with the empty vector B1 (lane 1). This reactivity will not affect the utility of the immune serum for detection of SERPIN B1 because it will be used with PMN lysates only.

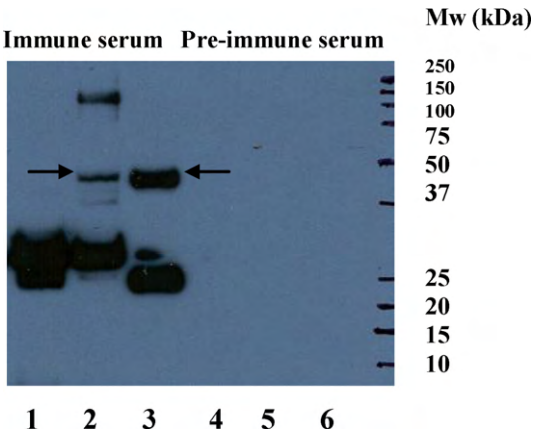

Fig. 3. Western blot analysis of ovine rSERPINB1 with polyclonal antibodies developed in mice. Lanes 1 and 4 represent lysate of *E. coli* transformed with the empty vector (without *SERPIN B1* insert). Lanes 2 and 5 represent lysate of *E. coli* transformed with the vector carrying the *SERPIN B1* insert. Lanes 3 and 6 represent rSERPIN B1 purified from the *E. coli* lysate on Nickel-NTA column. Lanes 1–3 were probed with the immune serum and lanes 4–6 were probed with pre-immune serum. Lanes 2 and 3 reveal the putative SERPIN B1 bands (shown by the arrows).

As stated earlier, the nucleotide and amino acid sequences of BHS and DS are 100% identical to each other. Therefore the putative difference in the control of the proteolytic activity of the PMN proteases in bighorn and domestic sheep is likely due to the difference in the level of expression of SERPIN B1 by the PMNs of BHS and DS. The rSERPIN B1 and the antiserum should help to determine the level of expression of SERPIN B1 by the PMNs of these two species.

Acknowledgements

This study was funded by the Wild Sheep Foundation, its state chapters and the Rocky Mountain Bighorn Society.

References

Benarafa, C., Priebe, G.P., Remold-O'Donnell, E., 2007. The neutrophil serine protease inhibitor serpin b1 preserves lung defense functions in *Pseudomonas aeruginosa* infection. J. Exp. Med. 204, 1901–1909.

Buechner, H.K., 1960. The bighorn sheep in the United States, its past, present, and future. Wildl. Monogr. 4, 3–174.

Cooley, J., Takayama, T.K., Shapiro, S.D., Schechter, N.M., Remold-O'Donnell, E., 2001. The serpin MNEI inhibits elastase-like and chymotrypsin-like serine proteases through efficient reactions at two active sites. Biochemistry 40, 15762–15770.

Coggins, V.L., 1988. The Lostine Rocky Mountain bighorn sheep die-offs and domestic sheep. In: Bienn. Symp. North. Wild Sheep and Goat counc., vol. 6, pp. 57–64.

Dassanayake, R.P., Shanthalingam, S., Herndon, C.N., Lawrence, P.K., Cassirer, E.F., Poter, K.A., Foreyt, W.J., Clinkbeard, K.D., Srikumaran, S., 2009. *Mannheimia haemolytica* serotype A1 exhibits differential pathogenicity in two related species, *Ovis Canadensis* and *Ovis aries*. Vet. Microbiol. 133, 366–371.

Fick, R.B., Naegel Jr., G.P., Squier, S.U., Wood, R.E., Bernard, J.E., Gee, L., Reynolds, H.Y., 1984. Proteins of the cystic fibrosis respiratory tract. J. Clin. Invest. 74, 236–248.

Foreyt, W.J., 1989. Fatal *Pasteurella haemolytica* pneumonia of bighorn sheep after direct contact with clinically normal domestic sheep. Am. J. Vet. Res. 50, 341–344.

Foreyt, W.J., 1994. Effects of controlled contact exposure between healthy bighorn sheep and ilamas, domestic goats, mountain goats, cattle, domestic sheep or mouflon sheep. Bienn. Symp. North. Wild Sheep and Goat Counc. vol. 9, 7–14.

Harlow, E., Lane, D., 1988. Antibodies a Laboratory Manual. Cold Spring Harbor Laboratory, 122 pp.

Jeyaseelan, S., Sreevatsan, S., Maheswaran, S.K., 2002. Role of *Mannheimia haemolytica* leukotoxin in the pathogenesis of bovine pneumonic pasteurellosis. Anim. Health Res. Rev. 3, 69–82.

Liu, W., Brayton, K.A., Lagerquist, J., Foreyt, W.J., Srikumaran, S., 2006. Cloning and comparison of bighorn sheep CD18 with that of domestic sheep, goats, cattle, humans and mice. Vet. Immunol. Immunopathol. 110, 11–16.

Rees, D.D., Rogers, R.A., Cooley, J., Mandle, R.J., Kenney, D.M., Remold-O'Donnell, E., 1999. Recombinant human monocyte/neutrophil elastase inhibitor protects rat lungs against injury from cystic fibrosis airway secretions. Am. J. Respir. Cell. Mol. Biol. 20, 69–78.

Remold-O'Donnell, E., Chin, J., Alberts, M., 1992. Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor. Proc. Natl. Acad. Sci. 89, 5635–5639.

Slocombe, R.F., Malark, J., Ingersoll, R., Derksen, F.J., Robinson, N.E., 1985. Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. Am. J. Vet. Res. 46, 2253–2258.